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Effect of Temperature, Sodium Chloride, and pH on Growth of *Listeria monocytogenes* in Cabbage Juice

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Human illness and death have resulted from the consumption of milk, cheese, and cole slaw contaminated with *Listeria monocytogenes*. Since the effects of temperature, NaCl, and pH on the growth of the organism in cabbage were unknown, a series of experiments was designed to investigate these factors. Two strains (LCDC 81-861 and Scott A, both serotype 4b) were examined. At 30°C, the viable population of the LCDC 81-861 strain increased in sterile unclarified cabbage juice (CJ) containing 0 to 1.5% NaCl; a decrease in the population of both strains occurred in juice containing ≥2% NaCl. At 5°C, the population of the Scott A strain in CJ containing up to 5% NaCl was reduced by about 90% over a 70-day period; the LCDC 81-861 strain was more sensitive to refrigeration but remained viable in CJ containing ≤3.5% NaCl for 70 days. Growth in CJ at 30°C resulted in a decrease in pH from 5.6 to 4.1 within 8 days. Death of *L. monocytogenes* occurred at 30°C when the organism was inoculated into sterile CJ adjusted to pH ≤ 4.6 with lactic acid. No viable cells were detected after 3 days at pH ≤4.2. At 5°C, the rate of death at pH ≤4.8 was slower than at 30°C. Growth of *L. monocytogenes* LCDC 81-861 at 30°C in CJ containing no added NaCl resulted in a titratable acidity (expressed as percent lactic acid) of 0.66%. Results indicate that strain LCDC 81-861 (a pathogenic strain isolated from cole slaw) is less sensitive to NaCl but more sensitive to refrigeration (5°C) than the Scott A strain (a human isolate). Both strains grew well at pH values that were lower than the minimum previously reported (pH 5.6).

Human illness and death have resulted from the consumption of milk (4), cheese (9), and cole slaw (11) contaminated with *Listeria monocytogenes*. The organism also has been isolated from rice soup with cream, lettuce, and meat products (8), indicating that a wide variety of foods can serve as vehicles for the transmission of listeriosis.

Recent outbreaks of food-borne listeriosis have generated much interest in defining the behavior of *L. monocytogenes* in food systems. Most research has been conducted with dairy products (2, 3, 9) because of their roles in recent outbreaks. In a 1983 listeriosis outbreak in Massachusetts, pasteurized milk was the suspected source of contamination (4), and in a 1985 outbreak in California, listeriosis was related to the consumption of soft Mexican-style cheese (9). However, sporadic outbreaks have occurred in which the vehicle of transmission was suspected to have been raw vegetables. In 1983, an outbreak of listeriosis in Canada was documented in which raw cabbage and cole slaw were the suspected sources of infection (11). *L. monocytogenes* serotype 4b was recovered both from an opened package of cole slaw from which a patient had eaten and from an unopened package from the plant. Moreover, although *L. monocytogenes* was not isolated from cold-stored cabbages harvested from fields fertilized with sheep manure, the sheep flock from which the manure had come experienced two deaths from listeriosis. In addition, Ho et al. (6a) cited lettuce and other raw vegetables as possible vectors of listeriosis. However, there is little available information concerning the growth response of *L. monocytogenes* in vegetables and vegetable products destined for human consumption. Therefore, the present study was initiated to determine the influence of temperature, NaCl, and pH on the growth of *L. monocytogenes* in cabbage juice (CJ).

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MATERIALS AND METHODS

Organisms and media. Two strains of *L. monocytogenes* (Scott A, a human isolate, and LCDC 81-861, a pathogenic strain isolated from cabbage) were investigated. Both strains were serotype 4b. The organisms were maintained on tryptic soy agar (TSA, pH 7.3; Difco Laboratories, Detroit, Mich.) at 5°C and activated for 24 h at 30°C in tryptic phosphate broth (TPB; pH 7.3), which contained, per liter of distilled water, 20.0 g of Proteose Peptone (Difco), 2.0 g of glucose, 5.0 g of NaCl, and 2.5 g of Na_2HPO_4.

Preparation of CJ. Fresh cabbage was obtained from a local market. Heads were chopped with a Silent Chopper (model 84142; Hobart Manufacturing, Troy, Ohio), ground in a stone mill (model M-MS-3; Morehouse Industry, Los Angeles, Calif.), set at 0.25 mm clearance, and pressed with a presser (type 60; Joseph Willmes, Bensheim Hessen, Federal Republic of Germany) at 5.5 kg/cm^2 pressure. CJ was collected after being passed through two layers of cheesecloth. The CJ was then dispensed into plastic containers and stored at −18°C until used.

Effect of NaCl and temperature. Quantities (1 l) of CJ were adjusted to 12 NaCl concentrations (0, 0.25, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, and 5.0% [wt/vol]). Samples (100 ml) of unsupplemented and NaCl-supplemented CJ were then dispensed into 250-ml Erlenmeyer flasks and autoclaved at 121°C for 15 min. Equal numbers of flasks of CJ containing each NaCl concentration were tempered at 30 or at 5°C.

Cultures of each strain grown for 24 h in TPB at 30°C and diluted 10^{-3} in 0.1 M potassium phosphate buffer (pH 7.0) served as inocula. Portions (1 ml) of inoculum were added to duplicate flasks at 30 and 5°C. After various incubation periods, viable populations (CFU per milliliter) were determined by serially diluting samples in phosphate buffer, making pour plates with TSA, and incubating the plates at

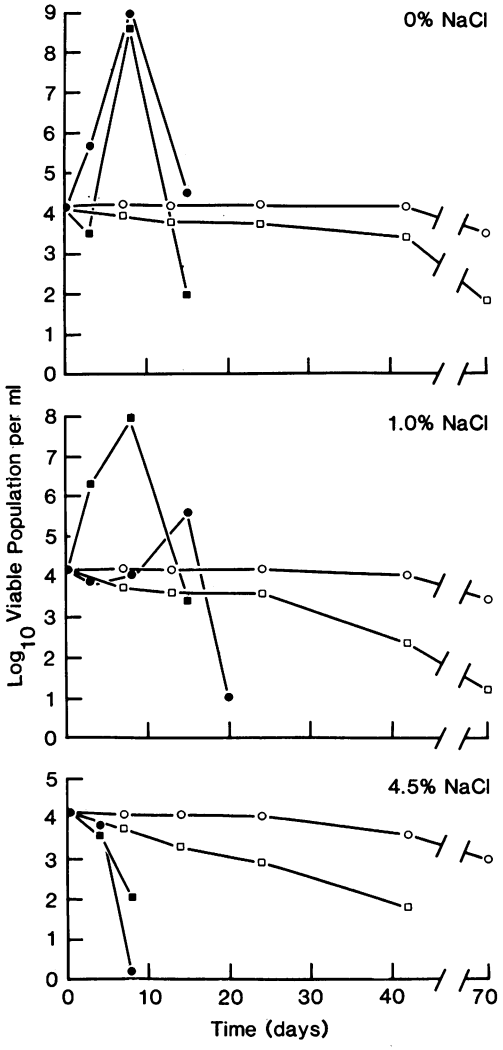

FIG. 1. Growth and survival curves for Scott A (○, ●) and LCDC 81-861 (□, ■) strains of *L. monocytogenes* in unclarified CJ supplemented with 0, 1.0, and 4.5% NaCl and incubated at 30 (solid symbols) and 5°C (open symbols).

30°C for 48 h. The pH of each sample was also determined at the time of sampling.

When no viable cells were detected in a particular sample with the above procedure, two tubes each containing 3 ml of sterile TPB were inoculated with 1 ml of sample; one tube was incubated at 30°C, while the other was incubated at 5°C for cold enrichment. At various incubation times, a loopful of each broth was streaked onto TSA. This culture was incubated for 2 days at 30°C and then examined for growth.

The effect of NaCl on the growth and fermentative characteristics of each strain in CJ was determined. Clarified CJ was obtained by autoclaving CJ at 121°C for 15 min, centrifuging it at 10,400 × *g* for 20 min, and collecting the supernatant. Once clarified, the CJ was supplemented with sterile NaCl (0, 0.5, 1.0, 1.5, and 2.0% [wt/vol]), dispensed in 9-ml quantities into sterile test tubes, and inoculated (10^2 CFU/ml). Over an 84-h period, A_{600}, viable population (CFU per milliliter), titratable acidity, and pH were measured. The A_{600} was measured with a Spectronic 20 (Bausch & Lomb, Inc., Rochester, N.Y.), and viable population was determined as described above. The titratable acidity (calculated as percent lactic acid) was determined by titration with 0.1018 N NaOH (10), and pH was monitored with a pH meter (model 805; Fisher Scientific Co., Pittsburgh, Pa.) equipped with a combination flat-surface electrode.

Effect of pH. Quantities (1 l) of sterile, unclarified CJ were adjusted to pH 3.8 to 5.6 in 0.2 unit increments with sterile 2.5 M lactic acid (Sigma Chemical Co., St. Louis). The samples were then dispensed into sterile 250-ml Erlenmeyer flasks, inoculated (10^4 CFU/ml), incubated at 5°C for 63 days or at 30°C for 21 days, and then analyzed for viable population and pH as described above. Unadjusted CJ (pH 6.1) served as a control.

RESULTS

Effect of NaCl and temperature. In unclarified CJ containing no added NaCl, the population of *L. monocytogenes* Scott A increased from 10^4 to 10^9 CFU/ml within 8 days when incubated at 30°C (Fig. 1). After 8 days, the population decreased rapidly, and no viable cells could be detected at 20 days. In CJ containing 0.25 or 0.50% NaCl, the population increased at 8 days and then gradually decreased over 24 days of incubation, at which time no viable cells were detected (not shown). However, in CJ containing 1% NaCl, an initial decrease in population observed at 3 days was followed by a gradual increase to $>10^5$ CFU/ml at 15 days. In the presence of 1.5, 2.0, 2.5, 3.0, 3.5, or 4.0% NaCl (data not shown), the viable population decreased continually until 15 days, at which time no viable cells were detected. At 4.5 and 5.0% NaCl, rapid inactivation of the population was observed, and no viable cells were detected at 8 days.

At 30°C, strain LCDC 81-861 was initially slightly more tolerant of NaCl than was strain Scott A. In the presence of 0.25, 0.50, or 1.0% NaCl, a consistent decrease in the population of strain Scott A was observed at 3 days, followed by an increase at 8 days to approximately 10^9 CFU/ml. In unclarified CJ containing 1.0 or 1.5% NaCl, the population of strain LCDC 81-861 increased to 10^9 CFU/ml after 8 days. At NaCl concentrations of ≥2.5%, viable populations declined to nondetectable levels within 15 days at 30°C. The rate of inactivation of strain LCDC 81-861 was slower than that of strain Scott A in 4.5 and 5.0% NaCl.

At 5°C, neither strain increased in population over a 70-day incubation period (Fig. 1), regardless of the concentration of NaCl in CJ. Furthermore, the rapid inactivation observed with ≥2.5% NaCl at 30°C was not observed at 5°C. Although cell division occurred at 5°C, as evidenced by increased turbidity, the viable population of strain Scott A remained constant (10^4 CFU/ml) in CJ containing 0 to 3.0% NaCl over 42 days of incubation, whereas the viable population of strain LCDC 81-861 decreased from 1 to 2 log cycles. In CJ containing 3.5 to 5.0% NaCl, the population of strain Scott A decreased by up to 90%. These observations indicate that the viability of both strains is influenced by refrigeration as well as by the additional stress effect of NaCl and that cellular division of *L. monocytogenes* can occur at 5°C in heat-pasteurized CJ containing up to 5% NaCl, even though the net number of viable cells may decrease with storage time. In another study (unpublished data), we observed that the viable population of *L. monocytogenes* increased on raw cabbage during the first 25 days of a 64-day storage period.

Transfer of 1-ml samples in which no viable cells could be detected to fresh TPB followed by incubation at 30 or 5°C (cold enrichment) failed to resuscitate cells; streaking the inoculated TPB incubated up to 42 days onto fresh TSA did not yield any colonies.

Growth of both strains in unclarified CJ with and without added NaCl resulted in substantial decreases in pH. At 8 days, the pH of samples in which heavy growth (10^8 to 10^9 CFU/ml) was observed had decreased from 5.6 to between 4.1 and 4.3. No pH change was observed in samples in which the population did not increase, indicating that growth of *L. monocytogenes* caused the reduction in pH. No pH change occurred in the samples incubated at 5°C.

Investigations to determine growth patterns of *L. monocytogenes* in clarified CJ supplemented with up to 2.0% NaCl indicate that both strains grow well at 30°C (Fig. 2). These results are in contrast to those obtained with unclarified CJ containing the same levels of NaCl. In those studies, ≥1.5% NaCl was lethal to both test strains. This suggests that in the presence of NaCl, the solid material in the unclarified CJ may impose a substantial inhibitory effect on *L. monocytogenes*. In clarified CJ, 2.0% NaCl slightly inhibited the rates of growth of both strains but was not lethal. For the LCDC 81-861 strain, the mean generation time (7) increased from 91.3 min in clarified CJ containing no added NaCl to 109, 108, 115, and 131 min in CJ containing 0.5, 1.0, 1.5, and 2.0% NaCl, respectively. Similar results were obtained with Scott A, which exhibited mean generation times increasing from 97 min in CJ containing 0% NaCl to 97, 108, 120, and 136 min in CJ containing 0.5, 1.0, 1.5, and 2.0% NaCl, respectively.

Both strains showed somewhat extended lag times in the presence of 1.5 and 2.0% NaCl (Fig. 2). Moreover, in the presence of 0, 0.5, and 1.0% NaCl, maximum population growth occurred within 44 to 48 h, whereas in CJ containing 1.5 and 2.0% NaCl, maximum population growth was not observed until 64 to 84 h of incubation.

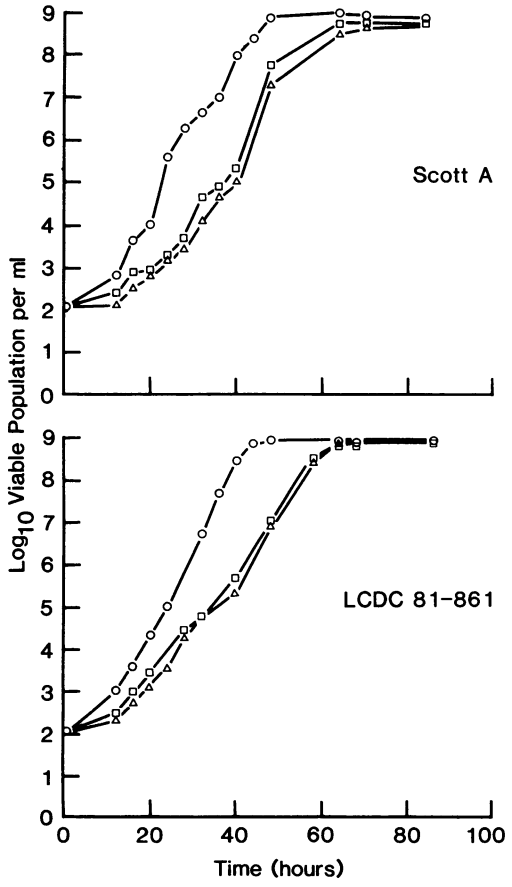

FIG. 2. Growth curves at 30°C for Scott A and LCDC 81-861 strains of *L. monocytogenes* in clarified CJ supplemented with 0 (○), 1.5 (□), and 2.0% NaCl (△).

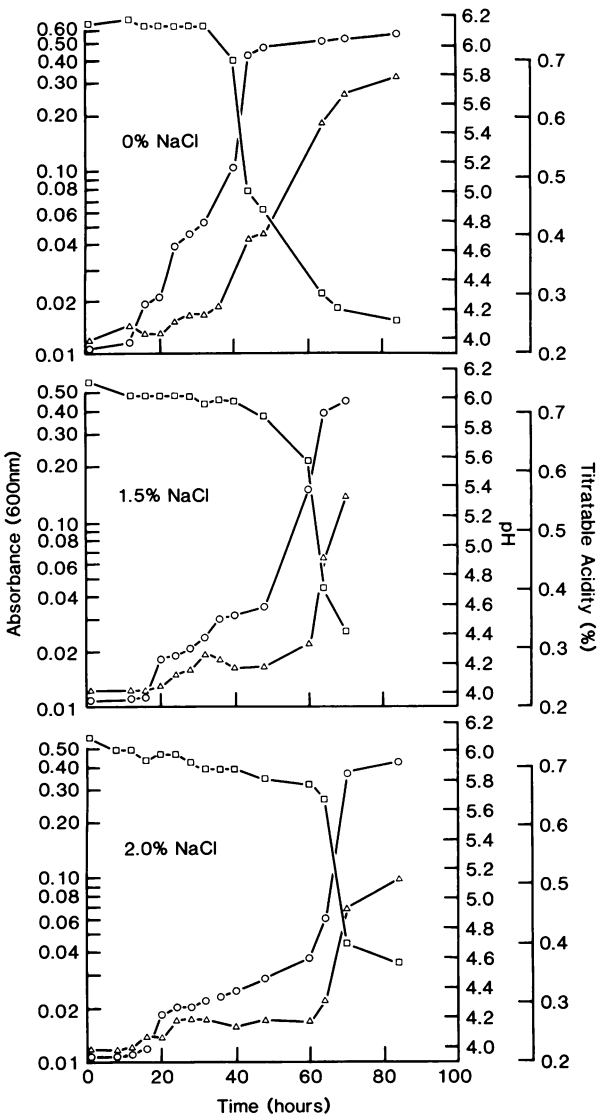

FIG. 3. Changes in A_{600} (○), pH (□), and titratable acidity (△) of clarified CJ supplemented with 0, 1.5, and 2.0% NaCl, inoculated with *L. monocytogenes* LCDC 81-861 and incubated at 30°C.

Changes in the pH and titratable acid of clarified CJ containing 0, 1.5, and 2.0% NaCl upon inoculation with strain LCDC 81-861 and were monitored over an 84-h incubation at 30° (Fig. 3). Similar results were obtained for the Scott A strain. Growth, as measured by increase in A_{600} of cultures, resulted in substantial increases in titratable acidity (percent lactic acid) and consequent reductions in pH. The lowest pH observed was 4.14, in CJ containing no added NaCl. Data indicate that an increase in acid content was associated with a population of 10^8 to 10^9 CFU per ml (Fig. 2). That is, no appreciable changes in titratable acidity or pH were observed in samples with populations of $<10^8$ CFU per ml. Acid production was reduced in the presence of 1.5 and 2.0% NaCl but was not affected by 0.5 and 1.0% NaCl (data not shown). In CJ supplemented with 0, 1.5, and 2.0% NaCl, 0.66, 0.56, and 0.51% lactic acid, respectively,

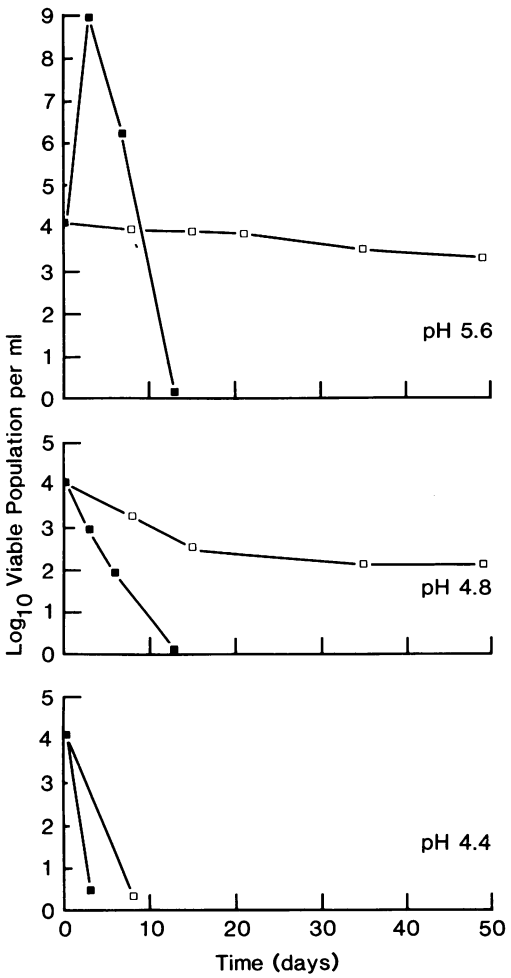

FIG. 4. Growth and survival curves of *L. monocytogenes* LCDC 81-861 in clarified CJ adjusted to initial pH 5.6, 4.8, and 4.4 with lactic acid. Cultures were incubated at 30 (■) and 5°C (□).

was detected after 84 h of incubation of strain LCDC 81-861. At the end of the incubation period, pH values of the CJ ranged from 4.14 (0% NaCl) to 4.56 (2.0% NaCl).

Effect of pH. When incubated at 30°C for 3 days, both strains of *L. monocytogenes* increased in population from 10^4 to approximately 10^9 CFU/ml of unclarified CJ, which had initially been adjusted to pH 5.4 and 5.2 with lactic acid. Growth in the control CJ (pH 5.6) gave the same results (Fig. 4). Rapid growth at 30°C in CJ initially at pH 5.6 was followed by rapid death of both strains. (Data for the Scott A strain are not shown.) In CJ initially at pH 5.0, the populations of the two strains remained constant for 3 days and then rapidly multiplied, reaching $>10^8$ CFU/ml at 7 days before declining. At pH 4.8, 4.6, and 4.4, cells were inactivated at 30°C; no viable cells were recovered beyond 14 days at 30°C. The rates of inactivation increased as the pH decreased. At pH ≤4.2, no viable cells of either strain were recovered upon sampling at 3 days.

Incubation of the pH-adjusted CJ at 5°C prohibited growth for up to 49 days of incubation (Fig. 4). At pH ≥5.2, the populations remained relatively constant (10^4 CFU/ml) for 22 days, whereas at pH <5.2, the populations gradually decreased during this period. Again, the rates of inactivation increased with decreasing pH. Viable cells were detected at 49 days in CJ initially at pH 5.0 and 4.8, whereas at pH 4.6 and 4.4 no viable cells were detected in 21-day samples. Both test strains remained viable ($>10^2$ CFU/ml) in CJ initially at pH 5.2 after 63 days of incubation at 5°C (data not shown). The rates of inactivation at pH 4.8, 4.6, and 4.4 were less at 5 than at 30°C. Evidently, lower temperatures may afford some protection to *L. monocytogenes* against the stress effects of low pH. No viable cells were detected in CJ at initial pH 4.2, 4.0, or 3.8 after 8 days of incubation at 5°C. The reduction of pH observed with growth of *L. monocytogenes* in CJ in previous experiments was also observed here.

When cells which had grown and reduced the pH of CJ to 4.3 were transferred to sterile clarified CJ adjusted to pH 4.6, 4.4, 4.2, 4.0, and 3.8 with lactic acid, no new growth was observed. This indicates that the cells had not adapted to low pH in this range. The reduction of the pH of CJ as *L. monocytogenes* grows may account for the rapid inactivation of cells observed in these and previous experiments.

DISCUSSION

Results of this investigation reveal that CJ is a good substrate for growth of the two tested strains of *L. monocytogenes* and support evidence that vegetables such as cabbages (11) could serve as vectors for the transmission of human listeriosis. The natural reservoir of *L. monocytogenes* has not been fully described, but the bacterium has been isolated from a variety of sources, including soil, feces, sewage, silage, and vegetation (1, 5, 6, 8, 12). In sporadic outbreaks of human listeriosis, the specific role of vegetables in transmission of the disease has not been clearly defined; however, in the Canadian outbreak it was suspected that cabbage fertilized with infected ovine feces resulted in contamination with *L. monocytogenes* and that extended cold storage of cabbage permitted proliferation of the pathogen (11). Although our results did not demonstrate proliferation of *L. monocytogenes* at 5°C, the bacteria survived very well in CJ, even in the presence of 5% NaCl. The potential for *L. monocytogenes* to persist and proliferate on vegetables and in brines used in processes to ferment vegetables is substantial.

Among the significant observations made in this investigation is that *L. monocytogenes* can tolerate and grow at pH less than 5.6, a lower value than that previously reported (6). In the present study, both strains tested grew well in CJ at initial pH 5.0 to 6.1. This pH range encompasses that of many vegetables as well as other low-acid foods. However, although more acidic environments (pH ≤4.8) were lethal, both strains were capable of reducing the pH of CJ to 4.14 before complete inactivation occurred. Thus, *L. monocytogenes* can proliferate in low-acid foods but would most likely be unable to tolerate the environment of acid foods and high-acid foods for extended periods of time.

Both strains were fermentative and produced substantial levels of lactic acid upon growth in CJ. This accounted for reductions in pH which most likely resulted in death and thus short stationary phases of growth. Both strains exhibited very little adaptation to low pH, further suggesting that a low-pH environment is quite inhibitory to *L. monocytogenes*. Gray and Killinger (6) state that it is nearly impossible to obtain viable cells transferred from cultures used in fermentation studies. This phenomenon was observed by us. *L. monocytogenes* is quite variable in its fermentative characteristics but can ferment glucose, levulose, trehalose, and salicin to acid (but not gas) (6). CJ, as well as other vegetable products, contains high levels of fermentable sugars such as glucose which can readily be utilized by *L. monocytogenes*.

The literature reveals little information concerning the behavior of pathogenic *L. monocytogenes* in food systems, particularly in vegetables and vegetable products. Results of experiments reported here demonstrate that CJ supports good growth of *L. monocytogenes* and that the organism is more tolerant of environmental stresses such as moderate NaCl concentrations and low-pH conditions than previously reported. Further research is needed to better identify other factors affecting growth and death of *L. monocytogenes* in foods, as well as the influence of these factors on the pathogenicity of *L. monocytogenes*.

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